MEMORANDUM

DATE: September 16, 2002

TO: Division of Shellfish Sanitation Staff

FROM: Robert E. Croonenberghs, PhD, Director
Division of Shellfish Sanitation

SUBJECT: Seawater - Procedure - Monitoring Program/Laboratory - Bacterial Source Tracking (BST)

Purpose

The objectives of this protocol are:

1. To establish stations and sampling procedure for the collection of seawater samples for bacterial source tracking (BST).

2. To establish the membrane filtering procedure to be used on seawater samples.

3. To establish the analytical procedures to be used in the laboratories for the culture and enumeration of *Escherichia coli* in seawater samples.

4. To establish shipping procedures for the shipping of sealed plates to MapTech, Inc.

Background on TMDL Reports and Implementation Plans

The Clean Water Act requires that states prepare plans for restoring the quality of polluted waters. These restoration plans are called *TMDL Reports*. The term *TMDL* stands for Total Maximum Daily Load, a mathematical modeling term that represents the total quantity of a pollutant that a water body can assimilate and still meet water quality standards. A TMDL report is the product of a special study to identify all sources of pollution contributing to the violation of water quality standards. For fecal coliforms causing shellfish closures, for example, run-off from pastures, failing septic tanks, leaking sewer lines and many other potential sources would be investigated. Once the fecal coliform sources have been identified, investigators calculate the amount of pollutants entering the stream from each source. Next, the reductions in pollutant loads needed to restore the stream to water quality standards are calculated. Finally, an *implementation plan* must be developed to identify specific pollution control measures that must be undertaken to restore water quality.

Public participation is an integral part of the TMDL development process. Citizens in the affected watershed are encouraged to attend public meetings where presentations are made on the findings of the TMDL study.

DEQ currently estimates that about 260 TMDLs have to be developed for shellfish closures and submitted to EPA by 2010, 50% of which have to be submitted by 5/1/06. The closures that must be addressed are those that were listed in the DEQ 303(d) List of 1998.

DSS Role in the Development of TMDLs

In order to determine the source of fecal coliforms to shellfish waters, the state and federal shellfish TMDL committee has decided to use antibiotic resistance analysis (ARA) on *Escherichia coli*, a member of the fecal coliform group. ARA is a process by which bacteria are tested for resistance against a battery of different antibiotics. Bacteria from pets, for example, tend to show resistance to different combinations of antibiotics than do cattle, humans, wildlife, birds, etc., hence the term bacterial source tracking (BST).

This variation in resistance can vary from watershed to watershed, thus fecal samples from known sources in the watershed have to be analyzed for ARA to compare against the seawater samples containing unknown sources. DSS will not be involved in the collection or initial enumeration of these fecal samples from known sources, the private lab running the ARA has that responsibility.

Since the Division is responsible for collecting the seawater samples used in classifying shellfish waters, and since the stations used for establishing TMDLs must be the same stations used to classify the shellfish waters, it is logical for the Division to collect the BST samples. DSS will then filter the seawater samples and enumerate the *E. coli* present, and attempt to achieve 20 - 80 colonies per plate. Plates will then be shipped to MapTech, Inc.

Seawater Stations to be Collected

The list of seawater stations to be collected will be updated each year and attached as an appendix to this working memo. It is anticipated that the 12 month sampling interval for a set of seawater sampling stations from various growing areas will run from September through August. Samples will be collected monthly on the last three hours of ebb tide.

Procedure for Filtering Seawater Samples

For each seawater sample station a 500 ml seawater sample shall be collected. The sample shall be collected using a disposable 500 ml sample bottle. The sample shall be placed on ice after collection and transported as soon as possible to the DSS field office laboratory. In the laboratory the sample shall be filtered through a 45 um membrane filters that are placed in 300 ml filter funnels. The quantity of water filtered for water samples collected during dry periods (no rainfall within the past 14 days shall) shall be in 4 – 100 ml aliquots. For rainy periods (any measurable rainfall within the past 14 days) there shall be 3-100 ml and 3-50 ml aliquots filtered and analyzed. These aliquots can be adjusted in order to obtain 20-80 colonies per plate. During extreme dry periods it may be difficult to achieve a count of 20 + colonies. In this case get as close to 20 colonies per plate as possible.

Procedure for Enumeration of *E. coli*

The QAQC protocol for the Division to follow, as submitted to DEQ, is attached as an appendix for reference.

Procedure for Logging Sample Analysis Results

Once the enumeration of *E. coli* colonies on the mTEC plates are completed these results shall be logged on the Shellfish Growing Area Water sample report form, which can be found in Appendix 3 in this memo. The total number of colonies on each replicate plate at each dilution shall be recorded. The number of organisms per 100 ml shall also be recorded as per the calculation stated at the bottom of the log sheet. The seawater sample collection field sheet shall be used to log the hydrographic data from the sample collection exercise.

If fecal samples are analyzed and *E. coli* colonies enumerated, the Fecal Sample log sheet shall be completed. This log sheet is identified in Appendix 4. The animal host of the fecal sample shall be identified along with the different dilutions and number of isolates from each sample.

A copy of the log sheet shall be sent with the E. coli plates to MapTech laboratory.

Procedure for Shipping Plates

One lab specialist from each office shall receive training in Department of Transportation Hazardous Materials Transportation. The *E coli* plates shall be placed in special cylindrical shipping containers designed for infectious substances (along with a cooler pack) and packed into the special shipping containers provided to each lab. The packing and transportation shall be done in accordance with 49 CFR 172 Subpart H. These containers should be shipped via overnight shipment using the account number supplied to each field office. The shipment shall be charged to that account and shall be identified on the shipping label.

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APPENDIX 1

BST Sampling Stations for September 2002 through August 2003

	Area #	Station #	Waterbody
White Stone			
	7	11	Shannon Branch
	7	14	White Point Creek
	7	19	Long Cove
	7	23, 27, 28.2	West Yeocomico River
	7	41	Mill Creek
	7	46, 47, 49	Lodge Creek
	7	51	Dungan Cove
	12	5, 6	Cockrell Creek
	30	2, 3	Whiting Creek
	30	7, 9	Meachim Creek
	34	7, 8	Healy Creek
	34	16	Wilton Creek
Norfolk			
	53	12, 14	Chisman Creek
	53	15Z4	Tabbs Creek
	53	17A	Topping Creek
	53	17.2A	Cedar Creek
	53	22	Patricks Creek
	53	28, 31	Poquoson River
	53	34	Lambs Creek
	53	36	Roberts Creek
	53	40	Lyons Creek
	53	44.2Z, 44.5	White House Cove
	53	47.5	Bennett Creek
	53	46.5Z	Easton Cove
	53	65	Back Creek
	54	5B	Front Cove
	54	17.2, 17.5	Northwest Branch Back River
	54	24	Southwest Branch
	54	29, 31	Harris River
	54	33W, 33Y	Wallace Creek
	54	34X	Inlet
	54	40	Long and Grundland Creeks
Accomac			
	76	11, 13	Messongo Creek
	76	19E	Young Creek
	76	19G	Guilford Creek
	80	4D	Finneys Creek
	80	7Z	Cedar Creek
	80	9A, 10	Onancock Creek
	80	14	Matchotank Creek
	84	13	Occohannock Creek
	90	9	Old Plantation Creek
	101	13	Chincoteague Channel/Fowling Gut
	101	47.5D, 47.5F	Assateague Channel/Sheepshead Creek

APPENDIX 2

Virginia Department of Health, Division of Shellfish Sanitation
Quality Assurance Plan

Laboratory support for BST analyses in selected Virginia shellfish growing areas

Table of Contents

1. *Project Description*

Introduction:

Use of bacterial source tracking (BST) methods for source identification is anticipated to be an integral part of TMDL development for shellfish growing areas. BST methods generally require cultivation and isolation of target bacterial genera. Because NSSP shellfish categorization programs utilize a fecal coliform standard, it is anticipated that TMDLs written for Virginia shellfish-growing waters will also be based on this indicator. BST methods evaluated for application to TMDL development in Virginia will use antibiotic resistance fingerprinting. Because of its location and time requirements for processing of water samples, VDH will culture and enumerate *Escherichia coli*, normally the dominant fecal coliform in water and scat samples, using EPA's recommended membrane filter methodology for marine waters. Membranes with adherent *E. coli* colonies will then be shipped to MapTech, Inc. for them to perform the *E. coli* verification and antibiotic resistance analysis.

Rationale:

Water samples collected for determination and/or recovery of *E. coli* should be processed within a given time period. The operational limit for determination of *E. coli* in estuarine waters is 24 hours. Initial processing of samples at VDH will provide compliance with the EPA method.

Tasks*:*

1. Provide sterile sampling containers for water and fecal source samples as required.
2. Process water samples to quantitatively recover *E. coli* using the EPA approved modified mTEC protocol (EPA/821/R-97/004) to enumerate *E. coli* based on typical colony characteristics. Multiple sample volumes will be used to obtain target density of 20-80 colonies/plate.
3. Incubate plates following EPA procedures.

**4. Count target colonies based on colorimetric response as described in EPA/821/R-97/004.
**5. Pack and ship membranes in sealed plastic dishes with ice packs to private labs. Laboratories require the following: 24 colonies/unknown; 12 colonies/known source (for library) for each sample. Shipments are required to conform to International Air Transport Association (IATA) regulations for transport of infectious agents. Shipping containers will be reusable and returnable by US surface mail.

2. *Project Organization and Responsibility*

Robert Wittman will have overall supervisory responsibility for the project.

Project supervisory personnel contact information:

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3. *QA/QC Objectives*

A laboratory quality control program will be maintained whose purpose is to insure practices that will remove or minimize sources of error in measurements. This includes following establishing specific protocols, calibration and maintenance of equipment, use of appropriate grades of reagents and chemicals, following of accepted protocols for good laboratory practices including record keeping and analytical QC checks, using established quantitative guidelines for data analysis, appropriate recording forms for data and other procedures, and providing for effective supervision and monitoring of the activities of technical personnel. Laboratory and quality assurance recommendations presented in *Standard Methods* (APHA 1999) sections 1020, 1060, 1080, 1090, 9020, 9030, 9040, and 9050 are followed in this laboratory.

**This study is designed to quantitatively recover (but not to confirm) *E. coli* from shellfish growing areas. Accordingly, we will follow QA/QC guidelines outlined in EPA/821/R-97/004 *Improved Enumeration Methods for the Recreation Water Quality Indicators: enterococci and Escherichia coli*, for section 10.3 Modified *E. coli* Method (USEPA, 2000) Performance factors for this method have been determined but as a quality assurance measure 1 station from each site surveyed monthly will be randomly selected and 10 colonies will be verified following procedures outlined in section 10.3.6 Verification Procedure in the document mentioned. Similarly, 5 colonies recovered from every 5^{th} source sample will also be confirmed using the verification procedure. All QA/QC recommendations for medium preparation, storage, and shelf life will be followed.

4. *Data Measurement Guidelines*

Data collected in performing the specific research tasks previously noted will be used to (1) to confirm the specificity of the modified mTEC procedure for recovery of *E. coli* from shellfish growing water samples and selected fecal sources in four different watersheds.

Comparability- The application of approved protocols for sample collection, sample processing, and analytical methods using accepted measurement units will provide data which shall be comparable between and within watershed sites.

Method Detection Limits- To insure that sufficient numbers of colonies are collected for BST analysis, each water sample will be analyzed in duplicate using three volumes to bracket predicted bacterial densities. Volumes chosen to process will be based on analysis of existing FC data collected by DSS, VDH.

5. *Sample Collection, Handling and Storage*

Water sample collection methods

General aseptic principles as outlined in *Standard Methods* (APHA 1999) should be followed. For this purpose sterile disposal 500 ml bottles will be provided. The bottles should be protected against contamination before, during, and after collection.

Surface water samples will be collected at a depth of 0.5 m. Keep the sample bottle unopened until immediately before filling and protect the container closure from contamination during sampling. To collect a sample securely clamp the bottle to the sampling pole provided with neck pointing downward at about a 30° angle. Remove the cap and plunge it neck downward below the surface. Rotate the bottle 180° so the neck points upward and allow it to fill completely. Direct the bottle away from the vessel to avoid contamination and sample "upstream" of the vessel motion. Securely close the bottle cap after sampling.

Water sample storage, processing time and accountability

Store samples in a closed insulated container. Ice is unnecessary and may even reduce counts during warm months owing to sublethal stress expressed during enumeration. Samples must be processed within 24 hours from the time of collection. Collection times and other information such as identities of persons responsible for collection; weather and water conditions at time of collection; and comments on unusual conditions or events observed should be entered in a field observation log. Times of receipt and collection will be recorded for each sample in a laboratory logbook.

Collection of supporting physical/chemical data

Interpreting microbiological data can require an understanding of the physical and chemical characteristics of the environment. Routine parameters measured or described during sampling of surface waters should include temperature and conductivity or salinity. Non-routine parameters such as stage and current of the tide and amount of precipitation should be useful for this study. In all instances, field instrumentation should be calibrated using appropriate standards and verified to be within specifications prior to and during sampling, and if required, after sampling is completed.

***6. *Recovery of E. coli using mTEC*

Modified mTEC agar will be used for recovery of *E. coli* from water samples as described in EPA/821/R-97/004 *Improved Enumeration Methods for the Recreation Water Quality Indicators: Enterococci and Escherichia coli* (USEPA 2000). Briefly, duplicate subsamples from each individual water sample are filtered through membrane filters using at least three volumes required to theoretically obtain 20-80 colonies per plate. The filters will be placed on modified mTEC agar and incubated with resuscitation following the aforementioned protocol. Modified mTEC agar uses a chromogenic glucuronide substrate and does not require additional biochemical testing for identification of presumptive *E. coli*. QA/QC procedures for verification of this method have been described above. Isolate confirmation is the responsibility of individual BST investigators. BST determinations should be performed on numerically-dominant *E. coli* isolates assuming an hypothesis that these isolates are dominant source. Filters will be selected for BST determinations starting from the smallest volumes processed in order to identify quantitatively dominant strains.

Membranes from water samples will be read after 22-24 hours as required. Presumptive *E. coli* densities on membranes from water samples will be evaluated to determine if appropriate volumes were selected.

***7 *Distribution of presumptive E. coli isolates to BST investigators*

Filters exhibiting approximately 20-80 colonies for each sample will be stored at 4°C after reading and shipped intact within sealed plates to the respective investigator by overnight shipping. Plates will be shipped with icepacks in approved biosafety boxes by overnight carrier and returned via regular mail.

8. Literature Cited

APHA. 1999. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, D.C.

USEPA. 2000. Improved Enumeration Methods for the Recreation Water Quality Indicators: enterococci and *Escherichia coli*. United States Environmental Protection Agency, Office of Science and Technology Washington DC 20460. EPA/821/R-97/004

APPENDIX 3

SHELLFISH GROWING AREA WATER SAMPLES

Shellfish Growing Area:________________________________ Date:_____________

Time samples received:____________________ 35 ºC Incubation started:___________

Samples filtered by:_______________________ Colonies counted by:_____________

Sample site	Sample code	*E coli* CFU per replicate volumes (mL)				E coli per 100 mL*
		100	50	30	5	

APPENDIX 4

FECAL SAMPLES

Shellfish growing area watershed: _____

Sample collection date: _____

Sample analysis date: _____

Sample ID	Animal Host	Dilutions	# of isolates	Homogenate frozen

Comments: _____